NEW YORK PATHOLOGICAL SOCIETY

ABSTRACTS OF PAPERS AND DISCUSSION

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Pathological Iron Metabolism in the Bone Marrow as Seen with the Electron Microscope

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Electron microscopy has established the erythroblastic island as a morphologic and functional unit of the bone marrow. A central reticular "nurse cell" appears to impart nutrients to surrounding rows of erythroblasts by the process of rhopheocytosis. Transfer of ferritin by this process is probably a passive phenomenon, since the amount transferred parallels the amount of iron present in the central reticular cell.

Ferritin is absent in iron deficiency, although rhopheocytosis remains prominent. Normally, all erythroblasts (proerythroblasts and normoblasts) and reticulocytes contain ferritin. Only the larger aggregates can be visualized by the Prussian Blue reaction in sideroblasts and siderocytes. Ferritin generally disappears when reticulocytes mature, even in hemochromatosis and infections, two conditions in which there is an excess of ferritin in erythroblasts. Interestingly, the increase in infections is entirely in the form of dispersed ferritin and cannot be visualized by the Prussian Blue reaction; i.e., sideroblasts are absent, in contrast to hemochromatosis where they are normal or increased.

It appears most likely that ferritin disappears from maturing reticulocytes because it is utilized for hemoglobin formation. It persists in mature red cells in Cooley's anemia, hypersideremic anemia with hypochromia and lead poisoning where hemoglobin formation is disturbed.

The origin of the ferritin in the "nurse cell" is a moot question. Isotopic iron studies indicate (Alpen) that siderophylin iron is immediately utilized by erythroblasts, while there appears to be a 12-to 24-hour delay of isotopic iron appearing in reticular cells. This evidence, and electron microscopic evidence, suggests that the iron of "nurse cells" is normally derived from intramarrow phagocytosis of red cells. In conditions of hyperferremia and following injection of iron compounds, iron can also be directly incorporated into ferritin of reticular cells.

The extent to which ferritin, by the process of rhopheocytosis, rather than siderophylin, provides iron for hemoglobin synthesis is also an unsolved question. In normal stages, some iron reutilization proceeds by way of erythrophagocytosis (which precedes hemolysis), formation of ferritin and rhopheocytosis. The major portion of reutilization of iron from destruction of or pathological red cells in the spleen involves transport to the marrow by diseryophylin (Noyes and others). Hemoglobin synthesis can certainly proceed without any visible ferritin, as in iron-deficiency anemia.

In certain pathologic states, accumulation of ferritin and related visibly dispersed or conglomerated iron micelles may point to the sites where hemoglobin synthesis or iron transport is blocked. In Cooley's anemia and the hypersideremic hypochromic (nonthalassemic) anemias, iron accumulates in the mitochondria, which are known to be involved in hemoglobin synthesis. In lead poisoning, the mitochondria are markedly abnormal and probably correspond to the

areas of punctate basophilia. However, the iron accumulates in other areas of the cell, suggesting a different type of block.

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DISCUSSION

GOETZ RICHTER: As, I am sure, all of us here have gathered, Dr. Bessis assigns a key role to the iron-containing protein, ferritin, in the iron metabolism of erythroblasts, and considers ferritin to be a source of the iron in hemoglobin. Moreover, he views a number of anemias in terms of presumed abnormalities, either in the utilization of iron derived from ferritin, or in an interference with ferritin synthesis. There must surely be reasons for the conspicuous presence of ferritin in erythroblasts and for the alterations in its disposition in these cells in various anemias—alterations demonstrated so elegantly by Dr. Bessis. How one interprets these phenomena really depends upon sequential arrangements of electron micrographs of bone marrow cells obtained from normal and from diseased persons at different times. Just as our concept of the maturation of leukocytes is derived from a composite of findings, physiological as well as morphological, so the idea that ferritin passes from reticulum cells to erythroblasts may in time come to rest on a composite of biochemical, physiological and morphological evidence.

The inferences concerning the existence of a specialized form of micropinocytosis, which Dr. Bessis has called "rhopheocytosis", are of interest from a general biological point of view. For if his interpretation of certain localizations of ferritin molecules at or near cell surfaces proves correct, we may assume that this process applies to other macromolecules as well. Does this process differ from the micropinocytosis which Palade has described in relation to vascular endothelium? There is also some evidence that macromolecules are moved out of cells by what might be termed "reverse micropinocytosis". Can one distinguish between entrance into and exit from erythroblasts when looking at pictures showing ferritin molecules in vesicles near the cell surfaces?

Can erythroblasts synthesize ferritin? There is ample evidence that many other types of cells can do so. Moreover, the most primitive erythroblasts are presumed to originate from reticulo-endothelial cells or mesenchymal cells of a kind likely to produce ferritin. Have erythroblasts retained the capacity to synthesize ferritin that less differentiated precursor cells may possess?

Dr. Bessis' findings on bone marrow cells from patients with thalassemia, hypersideremic nonthalassemic anemias, and other types of anemias are of interest because they have enlarged our knowledge of structural changes in cells under pathological circumstances, and because they suggest chemical impairments that may be part of the pathogenesis of these puzzling anemias. The presence of "micelles ferrugineuses" in mitochondria may well indicate an abnormal metabolic state.

The concept that the iron atoms in the hemoglobin of maturing erythroid cells are derived from ferritin is an attractive one, though results of work with radioactive iron have been interpreted as indicating that iron in hemoglobin comes more directly from the plasma. It is, of course, true that the physico-chemical state of iron in hemoglobin is different from that in ferritin. Yet one can envisage a transition from the one state to the other. One ferritin molecule, when fully loaded with iron, contains about 2400 atoms of iron in the form of ferric hydroxide, packed into a space roughly 60 Angstrom units in greatest dimension. By contrast, each hemoglobin molecule contains but four atoms of iron, and these are relatively widely separated. Thus, one ferritin molecule could furnish enough iron for about 600 hemoglobin molecules if conditions were right. A mechanism for such transitions could exist whether the ferritin of erythroblasts originates within these cells or not.

The Ultrastructure of Bone Cells

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The fine structure of cells in woven and lamellar bone using Araldite-embedded material is presented. The woven bone was obtained from a number of biopsies from patients with polyostotic fibrous dysplasia and the lamellar bone from the breast bones of 10- to 20-week old chicks. Osteoblasts have abundant endoplasmic reticulum, numerous vesicles, secretion droplets and long cytoplasmic processes which penetrate osteoid. Transition of osteoblasts into osteoid osteocytes and into osteocytes involves a successive reduction of cytoplasmic organelles. The collagen fibers in osteoid vary in diameter from approximately 100 A° on the surface to 600 A° in the deeper layers. Occasionally one notes early nucleation and partial mineralization of the collagen fibers in the osteoid layers.

The osteocytes and their processes are surrounded by a structureless material which may be a mucopolysaccharide. Osteoclasts differ markedly from other bone cells. They contain numerous nonmembrane-associated ribosomes and mitochondria suggesting a primary synthetic function. The brush border is a complex of long cytoplasmic processes between which apatite crystals are found. There is no phagocytosis of mineral. Frequently degenerating osteoclasts or portions of osteoclasts are seen. At resorption sites, no unmineralized collagen is seen and it appears that collagen is removed before or at the time of mineral solution. All bone surfaces are covered by a layer of cells. Where there is neither osteoid synthesis nor bone resorption, the bone surface has a layer of endosteal lining cells resembling osteocytes.

DISCUSSION

GOETZ RICHTER: Dr. Spiro's findings have indicated the extraordinary detail of fine structure in bone that is to be seen if painstaking, meticulous preparative techniques are employed.

Considerable attention has been paid in recent years to the mineralization of collagen in bone. One need only recall the earlier work of Robinson and Watson,[1] Sheldon and Robinson,[2] Fitton-Jackson,[3] and, of course, the studies of Glimcher and his group.[4] The orientation of hydroxyapatite upon collagen is apparently not a random one. Though the blood and all tissue fluids contain calcium and phosphate ions, apatite formation does not normally occur in collagenous tissue other than bone, cartilage or teeth. The lack of mineralization of collagen in nonosseous tissues has been ascribed to inhibition by substances, such as mucopolysaccharides, that are commonly present in the vicinity of collagen. According to Glimcher, the intra-molecular structure of collagen is so specialized that it can initiate nucleation of apatite crystals at specific sites in the collagen macromolecules.

The prominent rough-surfaced endoplasmic reticulum in osteoblasts, and the presence of what appear to be secretion droplets strongly suggest synthesis of protein constituents of bone by these cells. It has been suggested that osteoblasts secrete the monomer of collagen, and that the latter polymerizes in an extra-cellular location. There are, of course, various other proteins in bone, and some of these too have been presumed to originate in osteoblasts. The relative paucity of cytoplasmic organelles in mature osteocytes supports the concept that these cells are less active in the metabolism of bone. I was particularly interested in Dr. Spiro's observations on osteoclasts. Here the disperse form of ribosomes, i.e., those unassociated with membranes, is striking. It is known that in relatively undifferentiated cells the ribosomes occur mainly in this disperse form. Palade has pointed out that, as a rule, such cells are not secretory

in a strict sense, and that they lack the intricate membranous apparatus by means of which protein secretions are presumed to be exported to the extra-cellular environments. Yet it is known that such cells elaborate all sorts of proteins, notably enzymes. One wonders, then, just what activity that is specifically connected with metabolism of bone, is indicated by the electron micrographs. One thinks of enzymes related to resorption of bone, enzymes that might split proteins, or in some way change the pH in the vicinity of osteoclasts so that apatite might become soluble.

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The Use of Ferritin-conjugated Antibody to Localize Intracellular Viral Antigen by Means of Electron Microscopy

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Recognition of viral particles in thin sections of infected cells frequently presents difficulty. Generally speaking, intracellular virus can be identified with certainty only when particles similar in size and shape, and with characteristic internal structure are encountered in relatively large numbers. Moreover, there has been no way to identify and localize viral components before assemblage into recognizable viral particles. Recently, however, S. J. Singer (Preparation of an electron-dense antibody conjugate. *Nature (Lond.) 183*:1523-24, 1959) provided a means whereby antibody may be tagged for electron microscopy in a manner analagous to the immunofluorescence method employed for light microscopy. By using m-xylylene di-isocyanate he coupled ferritin with antibody globulin. Ferritin, containing a core of iron, is readily visible in the electron microscope and thereby permits the localization of individual antibody molecules.

Ferritin-conjugated antibody was employed in this laboratory initially to identify influenza virus and influenza antigens on the surface of infected cells. Subsequently, it was found that brief fixation in formalin, followed by freezing in a CO_2 alcohol mixture, rendered cells permeable to the conjugate while adequately maintaining cellular architecture and antigenic components. After immersion in ferritin-conjugated antibody the cells were washed, fixed in osmium tetroxide and processed for the electron microscope. Intracellular vaccinia virus has been tagged with specific ferritin-conjugated antibody, thus providing identification of the viral particles by immunologic means. Similar studies of cells infected with influenza virus have revealed dense intranuclear aggregates of viral antigen. It is suggested that this antigen is subsequently incorporated into the viral particles at the cellular surface.

DISCUSSION

JOHN KIDD: A number of the antigens with which viral antibodies react are so-called soluble antigens. They are quite distinguishable from the neutralizing antibodies. What are the ones you show, are they neutralizing antibodies? Secondly, there is an enormous variation in architectural density of the viral particles. What is responsible for this? What is inside the dumbbell-shaped forms? What accounts for the variation in architectural density?

COUNCILMAN MORGAN: To answer question one: To date we have used preparations of whole virus as antigens. It is certainly important to obtain antibodies to components of the virus and we are currently undertaking such an investigation.

In answer to your second question, the patterns of dark, sharply defined viral particles and less dense, ill-defined particles reflect the orientation of the crystalline lattice with respect to the plane of section—the virus appearing dense when central to the section, less dense when eccentric. The precise chemical composition of the internal components of vaccinia virus are not known.

On the Nature of Amyloid

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Amyloid proteins from the spleen of a patient having extensive amyloid deposition were extracted utilizing strong urea solutions. The extraction process was followed by assay for aromatic amino acids, tyrosine and tryptophane. The latter were used since amyloid histochemically gives a strong reaction for tryptophane. Examination of the residue by electron microscopy after several extractions in 6-molar urea and one in 8-molar urea revealed disappearance of the characteristic fibrils.

The extracted proteins contained total nitrogen concentrations in the usual protein range and total hexose ranging from 2 to 7 per cent. Ultracentrifugation revealed five components ranging in sedimentation coefficients from S-1 to S-23. Amino acid analyses revealed no cystine or hydroxyproline. Tryptophane was estimated to be about 3 to 4 per cent and tryrosine contents of the order of 5 per cent. Immunochemical analysis did not reveal any of the ordinary serum proteins. No antigenic component resembling gamma globulin could be detected, nor was there any evidence for the presence of c-reactive protein.

It is concluded that amyloid does not have ordinary antigen-antibody precipitate as a significant component in its composition. It may be that some of the protein classes seen in the ultracentrifuge represent polymers of a smaller protein. Such a capacity to polymerize would be consistent with the fibrillar formation characteristic of amyloid. It is hoped that it will be possible to reproduce *in vitro* fibril formation from some protein component or components extracted and purified this way, thus permitting more exact analysis of what appears to be a major and characteristic component of amyloid.